Metabolomics by *In Vivo* NMR

Editors

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New Haven, Connecticut, USA

John Wiley & Sons, Ltd

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West Sussex PO19 8SQ, England

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John Wiley & Sons Inc., 111 River Street, Hoboken, NJ 07030, USA

Jossey-Bass, 989 Market Street, San Francisco, CA 94103-1741, USA

Wiley-VCH Verlag GmbH, Boschstr. 12, D-69469 Weinheim, Germany

John Wiley & Sons Australia Ltd, 33 Park Road, Milton, Queensland 4064, Australia

John Wiley & Sons (Asia) Pte Ltd, 2 Clementi Loop #02-01, Jin Xing Distripark, Singapore 129809

John Wiley & Sons Canada Ltd, 22 Worcester Road, Etobicoke, Ontario, Canada M9W 1L1

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Library of Congress Cataloging-in-Publication Data

Metabolomics by in vivo NMR / editors, R.G. Shulman and D.L. Rothman.
p. ; cm.
Includes bibliographical references and index.
ISBN 0-470-84719-0 (cloth : alk. paper)
1. Metabolism. 2. Metabolism – Research – Methodology. 3. Nuclear magnetic resonance spectroscopy.
[DNLM: 1. Metabolism – physiology. 2. Cell Physiology. 3. Magnetic Resonance Spectroscopy – methods. 4. Muscles – metabolism. QU 120 M587 2005] I. Title: Metabolomics by in vivo nuclear magnetic resonance. II. Shulman, R. G. (Robert Gerson) III. Rothman, D. L. (Douglas L.)
QP171.M383 2005
572′.41′072 – dc22

2004013115

British Library Cataloguing in Publication Data

A catalogue record for this book is available from the British Library

ISBN 0-470-84719-0

Typeset in 10/12pt Times by Laserwords Private Limited, Chennai, India
Printed and bound in Great Britain by Antony Rowe Ltd, Chippenham, Wiltshire
This book is printed on acid-free paper responsibly manufactured from sustainable forestry in which at least two trees are planted for each one used for paper production.

This volume is dedicated to Lionel Trilling who taught us the excitement of thought.

‘The fascination of a growing science lies in the work of the pioneers at the very borderland of the unknown, but to reach this frontier one must pass over well traveled roads; of these one of the safest and surest is the broad highway of thermodynamics’, Lewis, G.N. and Randall, M. (1923) *Thermodynamics and the Free Energy of Chemical Substances*, McGraw-Hill, New York.

Contents

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Foreword

Bob Shulman and I were fortunate to be involved in the early developments of *in vivo* NMR. The field was beginning to open up, full of exciting promises, and competition between our two laboratories was healthy and desirable. At the same time during our friendly discussions we began to realize that our philosophies about the nature of this new approach were very similar and our experimental studies were complementary. For this reason, in 1984 we decided to write a joint contribution to a book, *Biomedical Magnetic Resonance*, edited by Tom James and Alex Margulis. Our article, 'Nuclear magnetic resonance of *in vivo* metabolism: from normal to pathophysiology', was published just 10 years after the first 31-phosphorus NMR study of intact isolated rat muscle and several papers from the Shulman laboratory on 13-carbon NMR studies on intact yeast cells. In our article we outlined 'how parallel and complementary developments in NMR spectroscopy in our two laboratories have helped to derive rich biochemical information from spectroscopic studies and to show how this information is beginning to have medical applications.' We noted that 'chemical energy is provided to the living cell by two primary biochemical pathways: glycolysis and oxidative phosphorylation' and that 'textbook description of these two pathways is likely to be largely correct, in contrast our ideas about molecular control of these events and the way they are co-ordinated with other functions such as energy demand, are largely speculative.' We also remarked that 'this is not surprising as understanding control requires that we should be able to study reaction fluxes, molecular interactions and metabolite distributions within the intact organism.'

Since 1984 the field has expanded enormously and our discussions became more wide-ranging and our friendship grew stronger. During a long walk in the woods surrounding the Max Grundig Clinic in Bühlerhöhe near Baden-Baden in 1994 Bob outlined his ideas for this book. I am delighted that the book on *Metabolomics by In vivo NMR* is now a reality. However, I am also pleased that it was not written earlier. The spectacular advances in genome sequencing and genomics in general in the last five or so years made it even more imperative that molecular networks, interactions and intracellular reaction fluxes be studied in the imaginative way that Bob Shulman and his colleagues developed the use of cellular NMR spectroscopy. I am therefore pleased about the timing of the book as it will have a more significant impact on future thinking about *in vivo* NMR as one of the important tools of the 'post-genomic' era. We not only need to understand cellular functions at the molecular level but also need to be able to bring the knowledge gained to solve problems in human disease. *In vivo* NMR spectroscopy and other forms of molecular imaging will bring clinical practice and biochemical understanding closer together. An example of this is the work reported by Bob in Bühlerhöhe under the title 'Nuclear Magnetic resonance studies of muscle and applications to exercise and diabetes' [*Diabetes* (1966) **45** (Suppl. 1): S93–S98]. This theme

is developed further in this book by Jerry Shulman, who shows how fundamental understanding of control of glycogenolysis and glucose metabolism in skeletal muscle could contribute to the clinical management of diabetes.

The DNA in the genome is the depository of biological information. Another level of cellular organization is responsible for the processing of this information and the 'metabolome', as emphasized in this book, provides the basis for the execution of the various cellular programmes. However, it is becoming increasingly evident that there is a complex network of interactions between these different organizational levels. The metabolic influence on transcription factor-controlled information processing is just one further piece of evidence for the need to look at cellular functions in a highly integrated way. A striking example of such interactions comes from the recent introduction of a new class of insulin-sensitizing drugs, the thiazolidines, that act as antagonists of the peroxisome proliferator activated receptor (PPARy), which is involved in the regulation of several transcription factors, including one for the expression of one of the mitochondrial uncoupling proteins. Serum free fatty acids (FFA) interact with this receptor. Thus understanding the relationship between obesity, diabetes, regulation of gene expression and energy provision and utilization is within our grasp. Biology has been transformed from an observational, descriptive science to a quantitative science. *In vivo* NMR as described in this book is on the path to this New Biology.

George K. Radda
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Introduction

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The wonderful advances of biochemistry, molecular biology and structural biology in the last half-century have created a foundation which *in vivo* NMR has utilized in the attack upon more complex organismic levels. Metabolic pathways, whose fluxes and intermediates are controlled by enzymes and genes in response to an organism's needs, are considered by some to be a worn-out topic of investigation – one that is largely 'complete'. The basic concepts are well established, the argument goes, and only minor details remain to be worked out. We feel that this view misses the most important point of reductionist biology – namely, that which has been established at one level can illuminate the more complex. For the past three decades scientists using *in vivo* nuclear magnetic resonance spectroscopy (MRS) to study metabolism have been developing an interdisciplinary vision for the future of biology at the molecular level. While their experimental findings and the immediate applications to particular metabolic pathways have been published extensively, the overall goals and philosophy of these individual studies have lurked, undeclared, below the scientific expositions. Only when these reports are considered together can one see that they build stepwise upon one another and ultimately provide a coherent direction for the future of biochemistry. We have had the pleasure of taking part in many of these studies and intend this book to serve as a review that outlines the theoretical framework that has been present in our minds but which has remained only implicit in our publications. Reviews are never complete and the progress they describe invariably changes, but we nevertheless are encouraged to assemble our report at this time. Our excitement stems from the depth and breadth of the results we wish to present. In the past 20 years many have asked, 'When are the biological results going to be worthy of the novel experimental methods developed for *in vivo* MRS?' In reviewing the literature for this book we are convinced that, to a communicable extent, the goal has been realized so that the use of *in vivo* MRS for the clarification of molecular events is well established. In addition to our enthusiasm, we are also motivated by a sense of obligation that arises from our conviction that biochemistry sits at a historic crossroads. Down one, now heavily traveled, path we see overwhelmed researchers chained to vast computers into which they feed ever-larger collections of descriptive data in the paradoxical hope that the only cure for the current information deluge is an

Metabolomics by In Vivo NMR. Edited by R. G. Shulman and D. L. Rothman
 ISBN: 0-470-84719-0

exponential increase in the number of data points. Down the other, less crowded, are researchers directly examining dynamic metabolic systems that use the genes, proteins and metabolites to realize biological functions. This book, in examining results from *in vivo* MRS studies of humans and animals, outlines ways in which this second path has been taken, and suggests how they might be applied more broadly to both experiments and ideology.

Practically, we propose that biochemistry can now reliably move upwards in complexity towards systemic physiology and beyond by making *in vivo* MRS studies of metabolic pathways. Biochemistry, like most biological fields, is intermediate between the reductionist explanations offered by chemistry and physics and the organismic functions studied by medicine, behavior or evolution. Unfortunately, it is much easier to simply collect more information about one particular level of complexity than to make the transition to the next. To evaluate such a relationship requires dynamic interplay between observations, new methodologies and entirely new concepts.

Biochemists are increasingly calling for more organismic insights – for 'functional genomics' and 'proteomics'. These visions, and the parallel medical hopes for genetic determinism, are in fact running headlong into the complexities of metabolism. Just as 'gene products' is a new word for enzymes, 'functional genomics', describing the functions served by gene products, is a new word for metabolism. In our view, *in vivo* MRS offers more informative ways of studying the metabolic pathways, and thus of moving from the relatively well-understood enzymes and metabolites to the organism-level phenomena of physiology and behavior.

In vivo MRS offers the opportunity to directly attach questions in functional genomics/metabolism, instead of pursuing these questions in a bottom-up (genetic) or top-down (cell biology) approach. 'Metabolomics' is a term with varied meanings but which most generally describes a large-scale, organized scientific attack upon the complexities of metabolism. It recognizes the importance of metabolism, and seeks to consolidate existing and newly generated information about metabolic pathways and constituents in the same way that proteomics endeavors to catalog biomolecular structures and protein expression profiles, and genomics addresses the vast arrays of DNA sequence information. In one generally accepted formulation, metabolomics plans to identify all the molecular components in the cell, including metabolites, macromolecules, cellular structures and solution conditions. It plans to use information available from proteomics and genomics to develop insights into organismic complexity. This book shares that goal but proposes that it can be pursued with MRS studies of metabolic fluxes *in vivo*, and with narrower but more realizable goals in mind (e.g. to understand organismic function at the level of physiology). By studying selected pathways with an eye for general principles, one can relate specific biochemical knowledge to insights about physiological processes. One result emerging in the following chapters is that, the more we understand the control of biochemical metabolism, the more clearly we understand how biochemical metabolism subserves physiological needs, how dependent biochemical control is upon physiology.

The unique perspective of this book derives from the strengths of ^{13}C NMR, a technique with the ability to measure metabolic fluxes in living humans and animals. *In vivo* methods for ^{13}C NMR detection are extensions of earlier developments in organic chemistry in which the resonance (or chemical shift) of the ^{13}C peak identifies a particular carbon of a particular molecule. For these assignments, sharp well-resolved resonances are required as observed for small molecules (MW $< 10^3$) and occasionally in larger molecules such as glycogen, where there are rapid internal motions. The natural abundance of the ^{13}C isotope is 1.1 % and it is NMR-visible, while the other ~99 % of the carbon nuclei are not NMR-visible. The low natural abundance allows two kinds of measurements: the first in which the ^{13}C NMR intensity allows the concentration of the compound to be calculated; and the second, which follows from increasing the ^{13}C fractional enrichment by introducing substrates, such as 1-^{13}C glucose, in which the ^{13}C enrichment has been increased towards 100 %. In this case, as the substrate reacts, the label moves down the biochemical pathway to subsequent compounds. From this measurement the flux through the sequence of reactions (i.e.

the pathway) can be calculated from the ^{13}C NMR intensities measured either as a function of time or measured when a steady state has been reached. These measurements provide the quantitative assessments of metabolite concentrations and rates of reaction, *in vivo*, which provide the unique, strong data sets that have allowed metabolism, studied this way, to provide a platform for novel insights into biological function.

In addition to the experimental opportunities offered by *in vivo* MRS, the study of metabolic pathways has been enriched by the development of metabolic control analysis (MCA). This analytic system provides a model in which *in vivo* MRS measurements of pathway flux and *in vitro* measurements of enzyme kinetics can be combined to make quantitative evaluations of the control of flux and of the concentrations of intermediates. The formal quantitative and theoretical aspects of MCA have been simplified in the past two decades so as to be readily applicable to *in vivo* MRS experiments. The essential simplified features of MCA needed to interpret the experiments discussed are presented in this book.

Three chapters introduce the book – this first chapter presents a unifying overview of the following sections. It delineates the roles proposed for *in vivo* MRS studies in physiology and medicine as interpreted and guided by MCA, and emphasizes the symbiotic strengths obtained by combining these two approaches. The second chapter summarizes the *in vivo* NMR techniques used in the subsequent chapters to derive the *in vivo* concentrations of metabolites and pathways fluxes. Although necessarily technical in nature, the explanations were written to be easily accessible to an undergraduate chemistry student. Chapter 3 outlines MCA, defining terms and presenting relations to be applied in several subsequent studies. MCA was invented more than 30 years ago, mainly by Kacser in Edinburgh, to explain the role of mutation in microorganisms. It defines the control of flux and the regulation of metabolite concentrations in terms of the measurable properties of the constituent metabolites and enzymes in a pathway. It represents a particularly valuable achievement of metabolomics, by deriving a functional role of these constituents *in vivo*. In establishing criteria for the control of flux by each enzyme in a pathway, it gives quantitative meaning to the concept of flux control, a concept whose differing definitions have led to confusion. The definitions and the parameters of MCA have been expressed as partial derivatives. This allows for valuable quantitation but, in light of the growing view that mathematics conceals knowledge, has undoubtedly has been responsible for MCA's neglect by biochemists. *In vivo* MRS experiments, which determine quantitative values of pathway fluxes and metabolite concentrations, are ideally suited to providing the sort of data MCA can then use to generate a coherent picture of metabolic systems. The simple formulations of MCA needed to take advantage of the MRS results are described in Chapter 3.

Chapters 4– 7 describe a metabolic system extensively studied by ^{13}C NMR and which focuses on glycogen synthesis, a valuable perspective for studying metabolic control in activity and disease. Chapter 4 reviews and integrates studies of glucose metabolism with a focus on the role of glycogen. These studies build upon the 100 % visibility of the ^{13}C resonances of this very large molecule – which allows concentrations and rates of synthesis to be obtained *in vivo* from the NMR spectra. The rate of glycogenesis in muscle as measured in non-insulin-dependent diabetic mellitus (NIDDM) subjects and their matched normal controls is described. The experiments provide understanding of the metabolic basis of glycogenesis and of the defects in NIDDM. They show that glucose after a meal is predominantly stored as muscle glycogen, that the rate of this pathway is reduced in NIDDM (thus explaining hyperglycemia) and that this reduced rate in NIDDM is caused by reduced recruitment of glucose transporters to the plasma membrane in response to insulin. This led to questions about the role of the enzyme glycogen synthase (GSase), the canonical example in biochemistry textbooks of an allosteric enzyme whose activity is under the control of a phosphorylation cascade. GSase, which is often considered to control the flux of glycogen synthesis as a result of its extensive modulation, is shown in Chapter 5 to not control this flux. Instead, Chapter 5 shows that GSase serves to maintain homeostasis, keeping the metabolic intermediates concentrations close to constant despite flux increases. Homeostasis is a physiological parameter, needed by other functioning elements in the body, so that this pathway property relates the flux through a pathway to systemic functions.

Furthermore the flux of glycogen synthesis itself serves homeostasis by maintaining blood glucose levels constant and storing the excess.

These results on the particular pathway of glycogenesis have implications that extend far beyond the specific pathway of muscle glycogen synthesis. The findings are relevant for other functional aspects of enzymes studied by modern biochemistry. Signaling pathways often express kinases, enzymes generally assumed to control fluxes by phosphorylating 'rate-limiting' enzymes in pathways. However, the combined function of allostery and phosphorylation in the archetypical phosphorylated enzyme, GSase, is shown not to be flux control of glycogen synthesis. Rather, it is to maintain metabolite concentration constant during changes in the flux of glycogen synthesis. This novel function for phosphorylation is an alternative to its generally accepted role of flux control, and is a broadly applicable finding of *in vivo* MRS as interpreted by MCA.

Chapter 6 describes muscle glycogen measurements during exercise. Topics include the depletion and restoration of glycogen stores during intense anaerobic exercise and moderate long-term aerobic exercise. Measurements of the change in total glycogen concentration in response to difference states of exercise were made by quantifying the natural abundance of ^{13}C NMR peaks. A particularly valuable measurement was made by infusing labeled 1-^{13}C glucose at a constant glycogen concentration in order to observe how different parameters affect glycogen turnover. The continual synthesis and consumption of glycogen during prolonged exercise has reinforced the notion that glycogen serves to supply the rapid pulse of energy needed during muscle twitches.

Chapter 7 reviews data on the synthesis and degradation of glycogen in the heart. It compares the results of ^{13}C MRS studies of myocardial glycogen metabolism with the skeletal muscle results, indicating similarities and differences. Heart data show that glucose uptake seems to be controlled by the glucose transporter/hexokinase entry step, as in muscle. Differences occur when the metabolic pathway bifurcates at G6P where the glycolytic flux is more active in the heart due to its ceaseless activity. The competition between these two paths for glucose flux and its influence by hormones and non-glucose substrates provide a recurring comparison between cardiac and skeletal muscle.

The next two chapters follow the existence and consequences of the rapid energy metabolism needed to support muscle contractions. Chapter 8 describes gated ^{31}P NMR measurements of phosphocreatine (PCr) in muscle. These data were acquired with a millisecond time resolution, so they allow for evaluation of the dynamic nature of muscle energetics. Specifically, by measuring the fall and rise of PCr during muscle contraction they provide a real-time basis for the energetics of muscle contractions. The usual values of energy consumption are obtained by comparing ^{31}P NMR measurements of PCr concentrations before and after a period of exercise lasting seconds or minutes. The gated NMR studies described here show how that approach severely underestimates the energy consumption because the PCr is substantially depleted and regenerated in synchrony with the contractions. The possibility that the rapid energy production must be provided by the anaerobic pathway leads to Chapter 9, which examines the role of lactate, the product of anaerobic glycolysis. The results disagree with the traditional views in which lactate is produced because of insufficient oxygen. Recent data have shown lactate appearances in well-oxygenated muscle, while the many recent functional magnetic resonance imaging results in the brain also show that lactate is produced during stimulation in well-oxygenated tissue. In Chapter 9 it is proposed that lactate is produced by the rapid anaerobic processes that respond to contraction. In this model lactate serves as a temporal buffer relating the millisecond need for energy to the long-term measurements of PCr glucose, glycogen and oxygen consumption.

In vivo ^{13}C and ^{31}P measurements were made on cellular suspensions several years before it was technically possible to study animal models and humans. Very detailed studies of energetic pathways in yeast and yeast spores made 20 years ago are juxtaposed with recent related studies in Chapters 10 and 11, and seen to provide novel answers to contemporary questions about metabolic adaptation to environment.

Differences and similarities of control mechanisms in yeast, described in Chapter 10, with activities of the same paths of glucose metabolism in human muscle, show the versatility of these shared energetic pathways. Central to the comparison is that, similar to mammalian pathways, which are shown in earlier chapters to be subservient to the organism's physiological needs, yeast can be subjected to rapidly changing environments (e.g. the sudden infusion of glucose, or the loss of nutrients) and has thus evolved mechanisms of survival despite such perturbations. In conformity with mammalian systems, yeast and yeast spores depend upon efficient energy usage during steady-state periods and are particularly well adapted to the cellular needs during transient periods when environments change rapidly.

Chapter 12 illustrates the great usefulness of isotopomer analysis. This method takes advantage of the fact that nearby ^{13}C sites in metabolites give spin-coupled NMR spectra. Labels at each site reflect the accumulated flux through different pathways so that measurements of the coupling can determine fluxes. This method does not require *in vivo* time course measurements and can be made at steady state either *in vivo* or, often, on extracts. Isotopomer experiments are made on body fluids or extracts with high-resolution NMR spectrometers. Their vertical magnets and rapid spectroscopic measurements are readily adapted to high throughput. Large data sets can be obtained and the specificity of metabolic flux determinations extended to large populations.

The last chapter summarizes results from the previous sections and discusses their relationship to general questions relevant to biological studies and to the goals implicit in metabolomics. The rapidly changing understanding of biochemistry and physiology based upon the new experimental approaches and guided by the formal structures of MCA, moves us confidently from molecules to biological function.